3,4-Dideoxyglucosone-3-ene as a mediator of peritoneal demesothelization

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Abstract

Background. The mesothelium contributes significantly to the functional, structural and homeostatic properties of the peritoneum. Bioincompatible peritoneal dialysis solutions contribute to mesothelial cell loss during chronic peritoneal dialysis. Cell death has been implicated in mesothelial cell loss, but the molecular mechanisms have not been adequately characterized. We now report the modulation of mesothelial cell death by the glucose degradation product 3,4-dideoxyglucosone-3-ene (3,4-DGE).

Methods. Human mesothelial cells were cultured from the effluents of stable dialysis patients. Apoptosis was quantified in cultured mesothelial cells and in peritoneal effluents. Confocal microscopy and inhibitors were used to assess molecular mechanisms.

Results. Peritoneal dialysis solutions with a high content of both glucose and glucose degradation products, but not those with low glucose degradation product content, induced mesothelial cell apoptosis and loss of cell viability in culture and in vivo. 3,4-DGE also induced mesothelial cell apoptosis. Apoptosis induced by peritoneal dialysis solutions and 3,4-DGE was associated with oligomerization of Bax at mitochondria and caspase activation. Bax antagonism prevented caspase activation, apoptosis and cell death. The pancaspase inhibitor zVAD was also protective.

Conclusion. 3,4-DGE and peritoneal dialysis solutions with a high content in glucose degradation products induce mesothelial cell apoptosis by a Bax-dependent mechanism. This could contribute to chronic demesothelization in peritoneal dialysis.

Keywords: apoptosis; bax; caspases; glucose degradation products; mesothelium peritoneal dialysis

Introduction

Chronic, long-term peritoneal dialysis (PD) is associated with the loss of the peritoneal mesothelial layer. This is thought to be related to the poor biocompatibility of PD solutions. Mesothelial cell loss may occur through detachment of live cells, mesothelial cell death and epithelial–mesenchymal differentiation [1,2]. Apoptosis is an active model of cell death of possible therapeutic relevance since it is regulated by the activation of intracellular lethal molecules [3]. Apoptosis has been implicated in biocompatibility both in haemodialysis and PD settings [4]. Apoptotic mesothelial cells are lost in the peritoneal effluent of PD patients [5]. An increased rate of apoptosis has also been observed in animal models of PD solution infusion [5] and PD solutions impair the reparative capacity of mesothelium [6]. PD solutions usually contain high concentrations of glucose and high concentrations of glucose induce apoptosis in certain cell types, such as the endothelium [7,8]. However, conventional glucose-containing PD fluids also have high concentrations of glucose degradation products (GDPs), which are produced during heat sterilization [9–11]. Newer, double- or triple-chambered PD solutions have a low-GDP content [10,11]. GDPs have been implicated in PD fluid mediated cytotoxicity [10]. In this regard, low-GDP, high glucose PD solutions did not display the cytotoxicity over leukocytes associated with high-GDP, high glucose PD solutions, suggesting a role for GDPs in cell injury [12]. 3,4-Dideoxyglucosone-3-ene (3,4-DGE) was identified as the main lethal GDP of conventional PD solutions and accounted for most of their lethal activity against both neutrophils and peripheral blood mononuclear cells [12,13]. Furthermore, 3,4-DGE also induces renal cell apoptosis [14] and may contribute to the better preservation of residual renal function associated with newer, low-GDP PD solutions [15].

We now report that 3,4-DGE accelerates apoptosis in human mesothelial cells from PD patients to an extent similar to conventional high-GDP, high glucose PD solutions and through similar molecular pathways.
Materials and methods

Patients

Mesothelial cell cultures were established from peritoneal effluents from six stable CAPD patients [1,16]. All patients were dialyzed with conventional glucose-containing, lactate-buffered PD fluids. Mean age was 66±11 years, four were females and two were males. Causes of renal failure included glomerulonephritis (n=3), polycystic kidney disease (n=1) and tubulointerstitial nephritis (n=2). Time on PD ranged from 2 to 12 months. Cells from each patient were treated with media and media containing the study products. Each independent experiment, and thus the n, represents cells from a different patient.

Mesothelial cell apoptosis in vivo was studied from freshly obtained peritoneal effluents. In a crossover design, five stable CAPD patients dialyzed with conventional glucose-containing, lactate-buffered PD fluids were studied. Mean age was 62±10 years, three were females and two were males. Causes of renal failure included glomerulonephritis (n=1), ischaemic nephropathy (n=1), polycystic kidney disease (n=1) and tubulointerstitial nephritis (n=2). Time on PD ranged from 3 to 12 months. They were treated for 2 weeks with conventional, single-chambered, glucose-containing PD solutions (Stay-Safe, Fresenius Medical Care, Bad Homburg, Germany) and then switched for another 2 weeks to double-chambered, glucose-containing (Balance Stay-Safe, Fresenius Medical Care) PD solutions. At the end of each study period the effluent from the overnight 2.3% glucose exchange and from a standardized 4-h 4.25% glucose dwell were assessed for apoptotic cells.

Studies were approved by the clinical ethics committee of Fundación Jiménez Díaz and patients provided an informed consent.

Mesothelial cell culture

After centrifugation of peritoneal effluents, cells were resuspended in a 5 mL culture medium, seeded into 25 cm² tissue culture flasks and incubated at 37°C in a humidified atmosphere with 5% CO₂. The culture medium, RPMI 1640 (Biowhitaker, Verviers, Belgium), supplemented with 20% foetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 2% Biogro-2 (containing insulin, transferrin, ethanolamine, and putrescine) (Reactiva, Madrid, Spain), was replaced every 4 days. Mesothelial cells were characterized by their morphology and by immunohistochemistry staining: positivity for cytokeratin, and negative staining for CD45 and von Willebrand’s factor [1,16].

Three different heat-sterilized, lactate-buffered PD solutions were studied: conventional, single-chambered, high-GDP 4.25% glucose (Stay-Safe, Fresenius Medical Care), double-chambered, low-GDP 4.25% glucose (Balance Stay-Safe, Fresenius Medical Care) and triple-chambered, low-GDP 4% glucose (Gambrosol-trio, Gambro, Lundia, Sweden) [11]. They are referred to throughout the text as conventional high-GDP PD solutions (4.25% glucose stay safe) or low-GDP PD solutions 1 and 2 (4.25% glucose stay safe) and 4% glucose (Gambrosol-trio, respectively). The 3,4-DGE concentration in conventional bags was 25–77 μM, depending on storage conditions.

Reagents

3,4-DGE was generously provided by Anders Wieslander (Gambro) [10,11,17–19]. Endotoxin levels were below the detection limit (Limulus amebocyte assay, Sigma) [14]. Formaldehyde (Sigma, St Louis, MO, USA) was used at 15 μM and 30 μM [12].

The pancaspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) (Bachem, Bubendorf, Switzerland) was used at concentrations (200 μM) previously shown to protect from apoptosis in human leucocytes and tubular cells [20,21]. zVAD-fmk was dissolved in methanol. Final concentration of methanol was 0.05% and it did not influence mesothelial cell apoptosis.

Bax inhibitor peptide P5 (Tocris, Ellisville, MO, USA) was used at concentrations (200 μM) previously shown to protect from apoptosis in tubular cells [14]. P5 is a cell permeable synthetic peptide inhibitor of Bax translocation to mitochondria. It was designed from Ku-70, a protein that suppresses the mitochondrial translocation of Bax and inhibits Bax-mediated apoptosis [22].

Assessment of cell death and apoptosis

Human mesothelial cells were cultured to subconfluence in 12-well plates (Beckton-Dickinson, Franklin Lakes, NJ, USA) and rested in serum-free media for 24 h. Cells were preincubated with inhibitory peptides or vehicle for 3 h and then PD solutions or serum-free RPMI 1640 with or without 50 μM 3,4-DGE, were added for 15 minutes. Then, the culture media were diluted 1:1 by the addition of RPMI 1640, in order to neutralize the acid pH and reduce the concentrations of glucose and GDPs and, thus, reproduce the physiological conditions during the PD exchange. The acid pH of conventional PD solutions was not found to contribute to apoptosis since pH neutralization did not reduce cytotoxicity.

For quantification of cell death, cells were harvested by pooling non-adherent cells with adherent cells, which were detached by gentle trypsinization. Apoptosis was quantified by flow cytometry assessment of DNA content of 5000 cells following permeabilization in buffer containing 100 μg/mL propidium iodide, 10 μg/mL RNase A, 0.05% NP-40 in PBS, as described [20]. The samples were analysed on a Facs calibur flow cytometer, using Cellquest software (BD Biosciences, San Jose, CA, USA). We must emphasize that, as cells were permeabilized, this method is not based on the ability of living cells to exclude propidium iodide. In each sample, the percentage of hypodiploid cells (decreased DNA staining: A₀), comprising apoptotic cells with fragmented nuclei, was counted [20]. Cell viability was quantified by the PMS/MTS test following the manufacturer’s information (Promega, Madison, WI, USA). Absorbance at 490 nm was monitored after colour development.

In peritoneal effluents, total apoptosis was quantified by assessment of DNA content as above. In addition,
mesothelial cell apoptosis was quantified by staining with the M30 cytodeath antibody (Roche Biochemicals) which recognizes a specific epitope generated by caspase cleavage of cytokeratin 18 that is not detectable in native cytokeratin 18 [23]. Cells were fixed with methanol at −20°C and incubated 1 h at room temperature with the FITC-M30 cytodeath antibody (1:250). Mean cell fluorescence intensity was quantified by flow cytometry. The specificity of the method to assess mesothelial cell apoptosis was studied in cultured mesothelial cells and peripheral blood leucocytes [12]. Cultured mesothelial cells or leucocytes were exposed to increasing concentrations of the apoptosis inducer staurosporin. Aliquots from the same well were stained in parallel with cytodeath and with propidium iodide to assess DNA content as above.

**Confocal microscopy**

Cells were plated onto Labtek™ slides in RPMI-20% FBS plus 2% Biogro. After 24 h the media were changed to RPMI-0% FBS and then cells were preincubated with inhibitory peptides or vehicle and cultured for 24 h in the presence of 3,4-DGE or conventional PD solutions as described above. Cells were fixed in paraformaldehyde 2% and stained with rabbit polyclonal anti-Bax (1:100, Santa Cruz Biotechnology), mouse polyclonal anti-cytochrome oxidase subunit IV (1:100, Molecular Probes Europe, Leiden, the Netherlands) or M30 cytodeath antibody (1:250, Roche Biochemicals, Mannheim, Germany) as previously described [21]. Cell nuclei were counterstained with propidium iodide.

**Statistics**

Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as mean ± SD. Significance at the P < 0.05 level was assessed by the Mann–Whitney test.

**Results**

**High-GDP, high glucose PD solutions and 3,4-DGE promote mesothelial cell apoptosis**

Conventional 4.25% PD solutions with a high-GDP content are cytotoxic to mesothelial cells and significantly decrease the number of viable cells at 48 h (Figure 1B). This toxic effect could be accounted for, at least in part, by 3,4-DGE, which also induces cytotoxicity with similar kinetics (Figure 1A, B). The decrease in number of viable mesothelial cells following a 48 h incubation with 3,4-DGE was dose-dependent within the range of concentrations reported in conventional PD solutions (Figure 1B). However, low-GDP PD solutions and another GDP, formaldehyde, are not cytotoxic to mesothelial cells (Figure 1B).

Since 3,4-DGE induces apoptosis in leucocytes [12], we studied whether the loss of cell viability could be associated with the occurrence of apoptosis. Indeed, contrast phase microscopy showed that 3,4-DGE treated plates had a lower number of attached cells and numerous detached, rounded, small-sized cells (Figure 1C). Detachment is an early feature of apoptosis and apoptotic cells shrink [3]. We then further characterized the occurrence of apoptosis by flow cytometry of DNA content (Figure 2), nuclear morphology (Figure 3), the appearance of cleavage products originated by caspases and the response to pharmacological modulation of the mediators of apoptosis Bax and caspases (Figure 4).

Flow cytometry of DNA content showed an increased number of hypodiploid cells, corresponding to apoptotic cells, in the presence of 3,4-DGE or conventional, high-GDP, 4.25% glucose PD solutions (Figure 2A). This technique was used to quantify apoptosis (Figure 2B). Induction of apoptosis by 3,4-DGE is dose-dependent (Figure 2B). Conventional, high-GDP, 4.25% glucose PD solutions induced apoptosis. However, increased lethality was not observed when cells were incubated with double- or triple-chambered PD solutions containing the same high concentration of glucose, but with a low-GDP content or with formaldehyde, a GDP which has been reported to retard remesothelization in vitro [24] (Figure 2A, B). These data support the notion that, as it is the case for leucocytes, GDPS, and in particular 3,4-DGE, contribute significantly to the toxicity of PD solutions against mesothelium.

**Bax is central to cell death induced by 3,4-DGE and high-GDP PD solutions**

Next, we studied the molecular mechanisms of apoptosis induced by 3,4-DGE and high-GDP PD solutions. In the mitochondrial pathway for apoptosis the usually cytosolic protein Bax oligomerizes at mitochondria and promotes the release of lethal molecules, which, in turn, lead to caspase activation. In control cells cultured in RPMI or in cells cultured in low-GDP PD solutions Bax remained diffusely cytosolic (Figure 3A). Either 3,4-DGE or high-GDP PD solutions were associated with the formation of Bax aggregates that preceded the development of nuclear apoptotic morphology features, suggesting that Bax aggregation preceded caspase activation at the individual cell level (Figure 3A). The Bax aggregates co-localized with cytochrome c oxidase IV, an enzyme that remains mitochondrial throughout the apoptotic process (Figure 3B).

In order to assess the feasibility of targeting Bax therapeutically in mesothelial cell death induced by 3,4-DGE or high-GDP, high glucose PD solutions, Bax function was antagonized by a Ku-70-derived peptide [22]. This intervention prevented the appearance of cytochrome fragments cleaved by caspases (Figure 4A) and decreased apoptosis (Figure 4B).

**Caspases are mediators of mesothelial cell apoptosis induced by 3,4-DGE and high-GDP PD solutions**

The release of lethal molecules from mitochondria may activate several death pathways. Activation of executioner caspases leads to the proteolytic cleavage of diverse substrates that may be used to characterize their activation. One such substrate is cytokeratin 8/18, whose caspase-cleavage product is identified by the M30 monoclonal antibody [25].
3,4-DGE and conventional, high-GDP PD fluids resulted in the appearance of cytokeratin 8/18 fragments cleaved by caspases (Figure 4A). A broad-spectrum caspase inhibitor (zVAD-fmk) prevented both caspase activation (Figure 4A) and apoptosis (Figure 4B).

Low-GDP PD solutions decrease the apoptosis of peritoneal effluent cells and mesothelial cells in vivo

We next studied peritoneal effluent apoptosis. Total apoptosis and mesothelial cell apoptosis were evaluated in conventional 2.3% glucose overnight effluents and 4 h 4.25% glucose exchanges and, following 2 weeks of low-GDP CAPD, compared with similar exchanges of low-GDP solutions in the same patient. Low-GDP PD solutions were associated with a lower percentage of total apoptotic effluent cells (Figure 5A) as well as a lower amount of apoptotic mesothelial cells (Figure 5B).

Cytodeath quantifies apoptosis in mesothelial cells but not in leucocytes. In cultured mesothelial cells stimulated to undergo different rates of apoptosis by exposure to different concentrations of staurosporin, there was a correlation between cytodeath-assessed cell death and cell death quantified by DNA content (Figure 5C). No cytodeath staining was observed in cultured apoptotic leucocytes (not shown).
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Fig. 2. Conventional PD fluids and 3,4-DGE induce mesothelial cell apoptosis. (A) Flow cytometry of DNA content disclosed that 50 µM 3,4-DGE and conventional high-GDP, high glucose PD fluids, but not newer low-GDP, high glucose PD fluids or formaldehyde (30 µM), promote apoptosis of mesothelial cells, detected as hypodiploid cells (arrows). (B) Quantification of mesothelial cell apoptosis by flow cytometry of DNA content. Conventional (high-GDP) and low-GDP (low GDP-1 and -2) high glucose PD solutions as well as 3,4-DGE and formaldehyde were studied for 48 h. *P < 0.005 versus control cells cultured in RPMI cell culture media, **P = 0.001 versus high-GDP PD fluid. Mean ± SD of four independent experiments.
Fig. 3. Molecular mechanisms of apoptosis induced by conventional PD solutions and by 3,4-DGE. (A) Bax aggregation at mitochondria. Cells were cultured in the presence of culture medium, 3,4-DGE, conventional high GDP, high glucose PD fluids or newer low GDP fluids (Low GDP-1 and 2) for 24 h. Bax and propidium iodide (PI) staining of nuclei. Observe the presence of Bax aggregates (arrows) in cells not yet displaying the apoptotic morphology. This is expected since Bax aggregation predates caspase activation. In addition, note the presence of apoptotic cell fragments characterised by shrunk, bright, pyknotic nuclear fragments (arrowheads) among cells treated with 3,4-DGE or high GDP PD solutions. Confocal microscopy, original magnification \( \times 120 \). (B) Colocalization of Bax aggregates and the mitochondrial marker cytochrome c oxidase IV illustrated by merging (arrows) in cells treated with 3,4-DGE or high GDP, high glucose PD solutions. Confocal microscopy, original magnification \( \times 200 \). Representative experiments of three performed.
Fig. 4. A Bax antagonistic peptide and the pan-caspase inhibitor zVAD prevent caspase activation and apoptosis induced by conventional PD solutions and 3,4-DGE. (A) Confocal microscopy. Cells were preincubated with P5 (Bax antagonist), zVAD (pan-caspase inhibitor), or vehicle and cultured in presence of control culture medium, 3,4-DGE (50 µM) or high GDP 4.25% glucose PD fluids for 24 h. Cytokeratin 8/18 fragments characteristic of cleavage by active caspases, and propidium iodide staining of nuclei. Note caspase activation in cells treated with 3,4-DGE or high GDP, 4.25% glucose PD fluids (arrows). Contrary to cells displaying Bax aggregation (Fig 3.A), most cells with cleaved cytokeratin also display already features of nuclear apoptosis (shrunk, bright, pyknotic nuclei). Cytokeratin cleavage is prevented by zVAD and P5. Original magnification ×120. Representative experiment of three performed. (B) Quantification of mesothelial cell apoptosis by flow cytometry of DNA content. Cells were preincubated with zVAD, P5 or vehicle and then cultured in the presence of control culture medium, 3,4-DGE (50 µM) or high GDP, 4.25% glucose PD fluids for 48 h. *p < 0.05 vs. 3,4-DGE, **p < 0.05 vs. high GDP PD fluid. Mean±SD of 4 independent experiments.

Fig. 5. Low-GDP PD solutions decrease the apoptosis of total peritoneal effluent cells and of peritoneal effluent mesothelial cells in vivo. (A) Quantification of total peritoneal effluent cell apoptosis by flow cytometry of DNA content (hypodiploid cells). Peritoneal effluents from the same patient were studied at baseline (high GDP) and following 2 weeks of PD therapy with low-GDP PD solutions. *P < 0.05 versus low GDP. (B) Quantification of mesothelial cell apoptosis by flow cytometry assessment of caspase-generated cytokeratin fragments. Results expressed as fold change over same patient results with a low-GDP PD solution because of patient to patient variability. *P < 0.02 versus low-GDP PD solutions. Mean ± SD of five patients. (C) Validation of cytodeath as a marker for mesothelial cell apoptosis. In cultured mesothelial cells induced to undergo different rates of apoptosis by different concentrations of the proapoptotic agent staurosporine for 24 h, cytodeath mean cell fluorescence correlates with apoptosis quantified as hypodiploid cells. Pearson correlation coefficient 0.93, P < 0.001.
Discussion

Mesothelial cells are lost in the course of long-term PD [26] due to the toxic effect of conventional PD solutions. Recent data point to a prominent role of GDPs in the toxicity of PD fluids. 3,4-DGE accelerates apoptosis in human leucocytes and is responsible for most of the cytotoxic potential of conventional PD solutions against these cells [12]. We now report that high-GDP, high glucose PD solutions and 3,4-DGE also induce mesothelial cell apoptosis, unravel the molecular mechanisms and identify possible therapeutic targets.

The concentration of 3,4-DGE in conventional glucose-containing PD fluids has been estimated to be from 25 to 125 μM [9,19]. At this range of concentrations 3,4-DGE induces apoptosis on cell targets, neutrophils and peripheral blood mononuclear cells that are relevant for peritoneal physiopathology [12]. 3,4-DGE also impairs the repair of the lost integrity of the mesothelial layer [24]. We now show that 3,4-DGE induces apoptosis in cultured mesothelial cells, although apoptosis kinetics differed from leucocytes suggesting different apoptosis regulation. The magnitude of apoptosis induced by 3,4-DGE is in the same range as that induced by conventional, high-GDP, high glucose PD solutions. Low-GDP/high glucose PD solutions did not promote mesothelial cell apoptosis, suggesting an important contribution of GDPs to the apoptotic effect of conventional PD solutions. Furthermore, mesothelial cell apoptosis induced by conventional PD solutions and 3,4-DGE share the same molecular pathway and the response to therapeutic agents. Taken together these results suggest that 3,4-DGE is an important mediator of cytotoxicity of conventional PD solutions against mesothelium. However, our studies do not exclude the possibility that other GDPs or combination of GDPs contribute to cell death.

Previous reports on mesothelial cell apoptosis induced by conventional glucose-containing PD solutions and/or GDPs did not explore the intracellular pathways for apoptosis and the concentrations of the GDPs that induced apoptosis were, in general, higher than those in PD fluids [27–29]. PD solutions and GDPs modulated the expression of caspase 9 and p53 [27]. However, caspase activation, the hallmark of apoptosis, was not studied [27]. 3-Deoxyglucosone (3-DG) had a marginal effect on mesothelial cell apoptosis: it required both the use of a high concentration (500 μM) and the concomitant presence of a high glucose concentration [28,29]. Only very high concentrations of methylglyoxal (500 μM) induced mesothelial cell apoptosis [29]. These data are consistent with early reports of GDP cytotoxicity, in which the then known GDPs, at concentrations found in PD fluids, were toxic [30]. High-GDP PD fluids contain 2–14 μM methylglyoxal [11,17]. Most reported values for 3-DG concentrations in conventional 4.25% glucose PD solutions were between 325 and 425 μM [9,11,31]. Interestingly, 3-DG is the precursor of 3,4-DGE and it is converted to 3,4-DGE in a temperature-dependent manner: the process is accelerated at 37°C, the temperature of cell cultures [32]. Our data thus expand previous observations that high-GDP, high glucose PD solutions induce mesothelial cell apoptosis by identifying the most toxic GDPs to mesothelium to date, by characterizing the intracellular pathways for cell death and by exploring mesothelial cell apoptosis in vivo.

Both 3,4-DGE and high-GDP, high glucose PD solutions activate the cell stress pathway that requires Bax. In this pathway Bax migrates from the cytosol to mitochondria, where it aggregates and permeabilizes the mitochondrial membrane, promoting the release of pro-death molecules that activate caspases [3]. The Bax pathway is also engaged in diabetes [8] and by 3,4-DGE in tubular epithelial cells [14]. The fact that the same death pathway is shared by diabetes, PD solutions and 3,4-DGE may be related to increased intracellular levels of GDPs in diabetics [33]. Despite numerous examples of cell type-specific death pathways our results suggest that therapeutic manoeuvres aimed at preserving mesothelial integrity may also protect kidney cells that share similar apoptotic pathways.

A lower total and mesothelial rate of apoptosis was observed in peritoneal effluents from low-GDP PD solutions-treated patients in vivo, supporting the relevance of the cell culture findings. The total rate of apoptosis most probably represents leucocyte apoptosis since leucocytes are the most abundant cells in the effluents [1]. By identifying the GDP responsible for most of the lethal effect and the molecular pathways involved, we provide clues to design therapeutic interventions if GDPs cannot be avoided and during short periods of time. Caspase inhibition preserves the viability of leucocytes exposed to PD solutions in cell culture and in vivo [12,20] and accelerate the clearance of bacteria [34,35]. Thus, it is conceivable that they will be used in the near future to boost antibacterial defences in different clinical settings. In addition to caspases, we have identified Bax as another target to preserve the mesothelium during acute insults.

In summary, our data suggest that a 3,4-DGE, a GDP present in conventional PD solutions, is responsible for a significant amount of mesothelial cell death induced by these solutions. Potential therapeutic targets to prevent peritoneal demesothelization have been identified that should be evaluated in vivo.

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